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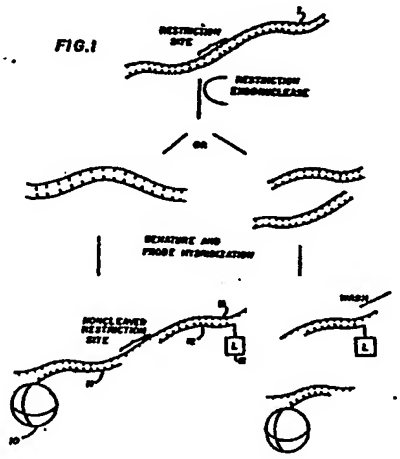
(54) Diagnosis of gene abnormalities by restriction mapping using a sandwich hybridization format.

(57) Assay for detecting the presence or absence of a particular genetic sequence in a nucleic acid strand. Double stranded sample nucleic acid is reacted with a restriction endonuclease whose activity depends on the presence or absence of the particular base sequence to be detected at the restriction site. The DNA is denatured and reacted with an immobilized nucleic acid probe and a labeled nucleic acid probe each of which hybridizes on either side of the restriction site. Separation of the aqueous and solid phases is effected and measurement of the label in either phase related to the presence or absence at the restriction site of the particular base sequence.

FIG.1



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DIAGNOSIS OF GENE ABNORMALITIES BY RESTRICTION MAPPING USING A SANDWICH HYBRIDIZATION FORMAT

Field of the Invention

This invention relates to assays in general, and in particular to those useful for detecting the presence or absence of a specific genetic sequence in a nucleic acid sample.

Background of the Invention

It has long been recognized that numerous disease states may be related to the presence of genetic abnormalities in an individual's chromosomes. Such genetic abnormalities often occur due to undesirable changes of nucleotide sequences in the nucleic acid strands carrying the genetic information. Transcription and translation of these erroneous sequences often results in nonfunctional proteins and in some instances, the abnormal sequences may actually prevent or inhibit transcription and/or translation. Consequently, identification of the presence of these abnormal genetic sequences is often diagnostically valuable.

It is an object of the present invention to provide an assay which is easily capable of identifying the presence or absence of specific genetic sequences associated with abnormalities.

Conventional methods for detecting specific sequences in DNA fragments have relied upon separation by gel electrophoresis. See Southern, E.M., "Detection of Specific Sequences Among DNA Fragments Separated By Gel

Electrophoresis", J. Mol. Biol. 98:503-517 (1975). Such methods have been employed for studying the structure of DNA and typically require reduction of the DNA into fragments which result from cleavage by restriction endonucleases. The fragments are separated by electrophoresis techniques in agarose or polyacrylamide gels, denatured and immobilized onto a membrane surface, and then hybridized with radioactively labeled single stranded DNA or polynucleotide probe as a means of identifying the thusly separated fragmentary pieces of sample DNA. Such methods are disadvantageously time consuming, labor intensive and are often incapable of identifying fragments of 500 nucleotide pairs or less depending upon the membrane surface or technique employed.

It is an object of the present invention to provide methods that have fewer steps and are accordingly simpler and less time consuming.

U.S. Patent No. 4,358,535 to Falkow et al., describes one class of methods directed to nucleic acid assays involving specific DNA probes in diagnostic microbiology. This method involves denaturization of the DNA and fixation thereof onto a support whereupon a labeled polynucleotide probe, specific for a DNA sequence, is contacted with the nucleic acid under hybridizing conditions. Hybridization of the probe to the single stranded nucleic acid is diagnostic of the presence of the pathogen. Such a method, however, fails to provide or teach how sequences having a limited number of differences distinquishing between an abnormal and normal gene can be detected. Although the Falkow technique could theoretically detect a single mutation, to do so would disadvantageously require the additional steps of culturing the bacteria, harvesting, and purifying the DNA.

It is an object of the present invention to employ DNA probes but in a far more useful format without reliance on methods requiring many steps and, in particular, to provide methods which can detect the presence of point mutations rather than identifying comparatively lengthy gene sequences.

Other methods of nucleic acid detection in samples have been described employing sandwich hybridization techniques and include for instance that described by Ranki et al., "Sandwich Hybridization As A Convenient Method For The Detection Of Nucleic Acids In Crude Samples", Gene 21:77-85 (1983). As with the Falkow et al. methods, the Ranki and related methods require hybridization of a nucleic acid probe with the sequences of interest and accordingly, rely upon the availability of such a probe having homologous sequences. Such methods are, however, generally only useful when large genetic sequences are sought to be identified such as for instance, the presence of cytomegalovirus DNA in clinical urine specimens. See Chou et al., "Rapid Detection and Quantitation of Human Cytomegalvirus in Urine Through DNA Hybridization", New England Journal of Medicine, Vol. 308, 16:921-925 (1983).

Antonarakis et al. described another method of genetic disease diagnosis in an article entitled "Genetic Diseases: Diagnosis By A Restriction Endonuclease Analysis", Journal of Pediatrics, Vol. 100, 6:845-856 (1982). This method involves the digestion of DNA with a restriction endonuclease, size separation by electrophoresis, denaturization, and Southern blot immobilization of the resultant single stranded fragments. Identification of the individual fragments is accomplished by hybridization with isotopically labeled single stranded DNA sequences and the hybridized sample then subjected to

autoradiography. Such methods disadvantageously require labeled probes to hybridize with the regions of interest following a general endonuclease digestion and size separation of the motley collection of DNA fragments.

It is an object of the present invention to instead employ a specific restriction endonuclease reactive at the site of interest only in special circumstances and DNA probes capable of hybridization with the remaining portions of the sample DNA thereby facilitating production of the necessary reagents specifically, and the detection method generally.

Recent investigations have revealed detailed information concerning the genetic structures associated with various disease states. Such information has been reported in a number of articles and include for instance, the following: Boehm et al., "Prenatal Diagnosis Using DNA Polymorphisms", New England Journal of Medicine, Vol. 308, 18:1054-1058 (1983); Pirastu et al., "Prenatal Diagnosis of β-Thalassemia", New England Journal of Medicine, 309, 5:284-287 (1983); Conner et al., "Detection of Sickle Cell β-globin Allele By Hybridization With Synthetic Oligonucleotides", Proc. Natl. Acad. Sci., 80:278-282 (1983); and Orkin et al., "Linkage of β-Thalassaemia Mutations And β-globin Gene Polymorphisms With DNA Polymorphisms In Human β-globin Gene Cluster", Nature, Vol. 296:627-631 (1982), all of which describe various genetic abnormalities associated with blood disorders. Another government funded source for more than 2700 DNA and RNA sequences is the centralized computer library called GenBank.

It is an object of the present invention to capitalize on the information revealed by such investigations,

specifically that concerning disease associated genetic sequences.

It is another object of the present invention to rely upon the vast array of restriction endonucleases which have recently become available.

It is yet another object to employ nucleic acid probes which may be immobilized or labeled in a hybridization scheme.

It is still yet another object to combine the aforementioned recently available reagents and knowledge in a hybridization format employing aqueous and solid phases whereby the presence of a single nucleic acid sequence change may be detected.

It is still another object of the present invention whereby the presence of point mutations in nucleic acid sequences may be detected with methods which measure agglutination or changes in agglutination patterns which may be related to the presence or absence of the nucleic acid sequence of interest.

Summary of the Invention

In accordance with the principles and objects of the present invention there are provided methods for determining the presence of specific genetic sequences in sample nucleic acid strands useful for diagnosing the presence or absence of disease associated genetic abnormalities.

The double stranded sample DNA to be analyzed is treated with a restriction endonuclease selected to operate at the site to be analyzed. As will be apparent to those

skilled in the art, the DNA will advantageously be of sufficiently high molecular weight and purity for the restriction endonuclease to be enzymatically active. The restriction endonuclease may be selected to enzymatically cleave the DNA only if the abnormality occurs, or enzymatically cleave the DNA only if the abnormality does not occur depending upon the nucleic acid sequence in those two cases and the concurrent availability of a suitable restriction endonuclease. Thus, intimate knowledge of the exact base pair sequence at the site of abnormality is required as well as the availability of a restriction endonuclease which operates at that site. Further, the restriction endonuclease will be advantageously selected so that it does not generally react anywhere else along the patient or sample nucleic acid, and specifically, elsewhere along the regions where probe hybridization will occur or regions intermediate thereof.

Activity of the restriction endonuclease at the site to be analyzed results in cleavage of the double stranded nucleic acid strand at that site thereby separating the DNA into double stranded fragments.

The double stranded DNA fragments are denatured to single stranded fragments and thereafter reacted with two nucleic acid probes. In the preferred mode, at least one of the probes is immobilized and is selected to hybridize with a base pair sequence on one side of the genetic site to be analyzed for, while the other nucleic acid probe is preferably labeled and selected to hybridize with a base pair sequence on the other side of said genetic site. Thus, the genetic site to be analyzed is intermediate the two probe hybridization regions of the sample nucleic acid. Since the sample DNA will be present in complementary single strands, the two nucleic acid probes will preferably also be provided in complementary forms to thereby

permit hybridization with both single-stranded sample DNA fragments and facilitate detection.

Immobilization of the nucleic acid may be accomplished by attachment to any of a variety of solid phase means including latex particles, plastic or glass surfaces, porous surfaces such as nitrocellulose fibers or the like. Similarly the labeled probe may have attached thereto a label selected from any of the well-known detectable labels commonly employed in immunoassays. These include, for instance, enzymes, fluorescent molecules, chromophores, reflective particles such as gold particles, magnetic particles, radioisotopes and the like.

The actual physical separation may be accomplished by any suitable means such as by washing, centrifugation and the like in accordance with well-known techniques.

Analysis of the presence or absence of the label with the immobilized phase or the aqueous phase will permit determination of the presence or absence of the specific genetic sequence analyzed for.

Alternately, both probes may be immobilized such as on microparticles or latex beads and agglutination or changes in agglutination patterns related to activity of the endonuclease, in turn dependent upon the presence or absence of the base pair sequence of interest.

Detailed Description of the Invention

Further understanding of the operation and principles of the invention may be had by reference to the Figure which schematically portrays the preferred method of the instant invention. The double stranded sample nucleic acid strand 1 is treated with a restriction endonuclease selected to

act at the site of interest. The logic relating the presence of label in the aqueous or solid phases with the presence or absence of the sequence to be analyzed for will be dependent upon the normal and abnormal base pair sequence as well as the availability of an endonuclease in either of those two circumstances. If not already apparent, this logic will become clear upon studying the Figure and the ensuing discussion.

As will be readily appreciated, activity of the restriction endonuclease will be, at least, partially dependent upon providing the sample DNA in a relatively high molecular weight and pure form. Accordingly, in some circumstances it may occasionally be necessary to perform additional preliminary steps to prepare the sample DNA to ensure adequate endonuclease activity.

The double stranded sample nucleic acid is then denatured and, treated either preferably simultaneously, or sequentially thereafter with each of two different nucleic acid probes 11 and 12. Denaturization of the double stranded DNA into single stranded DNA can be accomplished by heating an aqueous solution of DNA at elevated temperatures such as 90°C or greater or by exposing the DNA to an elevated pH such as 12 or greater. The resultant single strands are complementary and would undergo DNA renaturization if kept for a prolonged period at 65°C or under other appropriate conditions. Since there are two complementary copies, it will be advantageous to also provide complementary copies of probes 11 and 12 so that all available signal can be produced.

At least one of the nucleic acid probes 11 is preferably immobilized onto a solid phase surface 10, shown in the Figure as a latex bead or microparticle for convenience. The instant invention is not, however, so limited, and the solid phase may be any of a number of well-known formats.

These formats include, for example, latex beads of any size including the so-called macroscopic and microscopic sizes; plastic or glass surfaces such as the walls of a microtiter well, test tube and the like; porous surfaces such as the fibers in a cellulose mat employed in conventional blot hybridization techniques or any other similar structure. The only criteria is that if one of the probes is to be labeled and therefore soluble, the solid phase portion of the assay must be separable from the aqueous phase of the assay whereby the label can be detected in the aqueous phase if cleavage has occurred at the restriction site. Otherwise, if agglutination or agglutination changes are to be used to monitor endonuclease activity, then both probes will preferably be labeled with latex beads, microparticles, or the like.

Continuing with the description of the preferred mode, probe 12 is suitably labeled with label 13 which permits its ready identification and/or quantitation in either the aqueous or solid phase portions of the assay. Such probes will preferably be fluorescent, enzymatic, or isotopic in nature in order to provide the desired sensitivity, however, other labels are also contemplated and may include metallic particles, chemiluminescent or phosphorescent molecules, non-fluorescent dyes and the like. These labels can be readily detected with great sensitivity by employing appropriate instrumentation readily available commercially.

With further reference to the figure, Probes 11 and 12 will also be suitably selected in order to hybridize with the single stranded patient DNA sample on either side of the restriction site. Thus, if no endonuclease digestion occurred due to an incorrect sequence (vis-a-vis the enzyme) at the restriction site, the immobilized probe, hybridized on one side of the site, will be connected to

the labeled probe, hybridized to the sample on the other side of the restriction site.

Separation of the solid and aqueous phases may then be effected by any of a variety of means which may include, for instance, a wash step such as indicated in the Figure whereby the aqueous phase containing soluble reagents is removed. Of course, other methods of separating the two phases including centrifugation etc. are equally applicable and may be selected in accordance with the investigator's expertise, available equipment, or type of insoluble phase chosen.

Following separation, either the solid phase portion or the aqueous phase portion may be examined for the presence of label. Presence of label associated with the solid phase would be indicative of little or no restriction endonuclease activity as would the absence of label in the aqueous portion. As should be readily apparent, presence of the label in the aqueous portion (or absence thereof in the solid phase portion) would therefore be indicative of restriction endonuclease activity at the cleavage site. Based on the restriction site sequence and the selected restriction endonuclease, presence or absence of the label in the solid or aqueous phases may then be related to the presence or absence of the normal or abnormal nucleotide sequence as aforesaid.

Alternately, if agglutination detection techniques are to be employed, then the separation step need not be performed. Instead the aqueous solution can be examined directly for agglutination, i.e., no enzymatic digestive activity, or still alternately, changes in agglutinate settling rates, etc., for determination of enzymatic digestive activity.

As may now be readily perceived, in order to fully benefit from the instant invention, the sequence of base pairs at the site of interest must be known or determinable. As previously indicated, many investigators have been successful in identifying disease associated genetic abnormalities and in particular, the nucleotide sequences associated therewith. Knowledge of these sequences allows the selection of an appropriate restriction endonuclease. Endonucleases are commonly available commercially from a variety of manufacturers. With the continued success in identifying disease associated nucleic sequences as well as the expected discovery of still additional restriction endonucleases, the versatility of the instant invention will continue to increase.

Similarly, the nucleic acid probes will be selected, or produced using methods now well-known, so as to hybridize with (i.e., be substantially homologous with) the single stranded nucleic acid on either side of the site of interest. One commercial supplier of labeled nucleic acid probes is Enzo Biochem of New York, New York.

The instant invention can also be utilized in a variety of oncogene studies such as assisting the identification of the bacteriophage lambda of the genomic library which carries the oncogene responsible for transformation of normal cells into tumor cells. For example, the bladder carcinoma oncogene is believed to comprise approximately 5,000 DNA base pairs. Similarly, the proto-oncogene which is the normal antecedent of the oncogene, is approximately the same size but has no transforming effect associated therewith. Since the proto-oncogene is conserved, one may conclude that there must be some associated organismic function although as yet unidentified. The proto-oncogene, however, differs from the oncogene in only one

base pair. Such a difference could arise from a point mutation wherein the guanine base is replaced with a thymine. Alternatively, the replacement may occur by chromosomal rearrangement, gene amplification or enhanced transcription. In any case, the methods and principles of the instant invention would permit identification of the oncogene versus the proto-oncogene given the appropriate restriction endonuclease having activity at the point mutation for either the oncogene or the proto-oncogene.

Equally contemplated is the use of oligodeoxynucleotides (also known as oligonucleotides) for at least one of the nucleic acid probes. Oligonucleotides typically contain at least approximately 14 base pairs as opposed to the more conventional probe size of at least approximately 50 base pairs. With shorter probes, lower melting points will be observed and, as with the longer probes, homology can be gauged based on changes in the melting temperatures albeit at lower temperatures. The shorter probes can permit the selection of probes which hybridize with the sample DNA over areas which encompass or overlap the restriction site. In these instances, the manner of endonuclease cleavage and probe hybridization must be carefully chosen so that if digestion at the restriction site occurs, hybridization of the probe does not take place. Thereafter, the logic relating the presence of label in the aqueous or solid phases with the presence of a particular base sequence at the restriction site proceeds as previously described. Since these conditions, even with very short probes, will be difficult to attain, the aforementioned embodiments utilizing conventional probes homologous to sequences not overlapping the restriction site are preferred.

Various alternatives to the aforementioned, including the rearrangement of steps and other similar variations, will

become readily apparent to those skilled in the art, however, all such variations are to be deemed equivalents within the spirit and scope of the present invention and as defined by the following claims.

CLAIMS

1. A method for determining the presence of a specific base pair sequence occuring within a nucleic acid strand in an aqueous sample comprising:

a) providing an insolubilized nucleic acid probe homologous to a first base pair sequence on one side of said specific base pair sequence;

b) further providing a labeled nucleic acid probe homologous to a second base pair sequence on the other side of said specific base pair sequence;

c) contacting said nucleic acid strand either with a restriction endonuclease which will cleave said nucleic acid strand at said specific base pair sequence if present but not at any other site between or along said first and second base pair sequences whereby said nucleic acid strand is digested into at least two portions if said specific base pair sequence is present or with a restriction endonuclease which will not cleave said nucleic acid strand at said specific base pair sequence if present nor at any other site between or along said first and second base pair sequences whereby said first and second base pair sequences remain connected if said specific base pair sequence is present;

d) denaturing said nucleic acid strand into single stranded nucleic acid after said contacting step;

e) reacting said single stranded nucleic acid with said insolubilized nucleic acid probe and said labeled nucleic acid probe under conditions which permit hybridization to occur;

f) separating said insolubilized probe from said aqueous sample; and

g) determining the presence of said specific base pair sequence on the basis of whether or not said

labeled probe is associated with said immobilized probe or with said aqueous sample following said separation step.

2. The method of claim 1 wherein the restriction endonuclease cleaves at said specific base pair sequence, in which said determining step comprises either detecting the presence or absence of label associated with said immobilized probe and relating said presence or absence to an absence or presence of said specific base pair sequence respectively or; detecting the presence or absence of label in said aqueous portion and relating same to a presence or absence of said specific base pair sequence respectively.

3. The method of claim 1 wherein said restriction endonuclease does not cleave at said specific base pair sequence, said determining step comprises either detecting the presence or absence of label associated with said immobilized probe and relating said presence or absence to a presence or absence of said specific base pair sequence respectively or; detecting the presence or absence of label in said aqueous portion and relating same to an absence or presence of said specific base pair sequence respectively.

4. The method as provided in any one of claims 1 to 3 wherein said specific base pair sequence is associated with a genetic abnormality in turn associated with a disease condition whereby the determination of the presence of said genetic abnormality constitutes diagnostic evidence of said disease.

5. The method as provided in any one of claims 1 to 4 wherein said insolbilized nucleic acid probe is immobilized by attachment to a solid phase selected from the group consisting of microbeads, latex particles, plastic surfaces, glass surfaces and porous surfaces; and said label is selected from the group consisting or radioisotopes,

fluorophores, chromophores, enzymes, magnetic particles, light scattering particles and reflective metallic particles.

6. The method as provided in any one of claims 1 to 5 wherein said insolubilized nucleic acid probe is immobilized on nitrocellulose paper and said label is ^{131}I, ^{32}P ^{125}I, of ^{35}S.

7. The method as provided in claim 6 wherein the label is ^{32}P.

8. The method as provided in claim 6 wherein the label is ^{125}I.

9. The method as provided in any one of claims 1 to 8 wherein said separation step is selected from the group consisting of washing and centrifuging.

10. The method as provided in any one of claims 1 to 9 wherein at least one of said first or second nucleic acid probes is an oligodeoxynucleotide homologous to a base pair sequence on said sample nucleic acid strand which includes said restriction site and wherein the binding properties of said oligodeoxynucleotide with its homologous base pair sequence will be detectably altered with restriction endonuclease digestion at said specific base pair sequence.

11. A method for determining the presence of a specific base pair sequence occurring within a nucleic acid strand in an aqueous sample comprising:

a) providing a first insolubilized nucleic acid probe homologous to a first base sequence on one side of said specific base pair sequence;

b) further providing a second insolubilized nucleic acid probe homologous to a second base sequence on the other side of said specific base pair sequence;

c) contacting said nucleic acid with a restriction endonuclease which will cleave said nucleic acid at said specific base pair sequence if present but not at any other site between or along said first and second base pair sequences whereby said nucleic acid is digested into at least two portions if said specific base pair sequence is present;

d) denaturing said nucleic acid into single stranded nucleic acid after said contacting step;

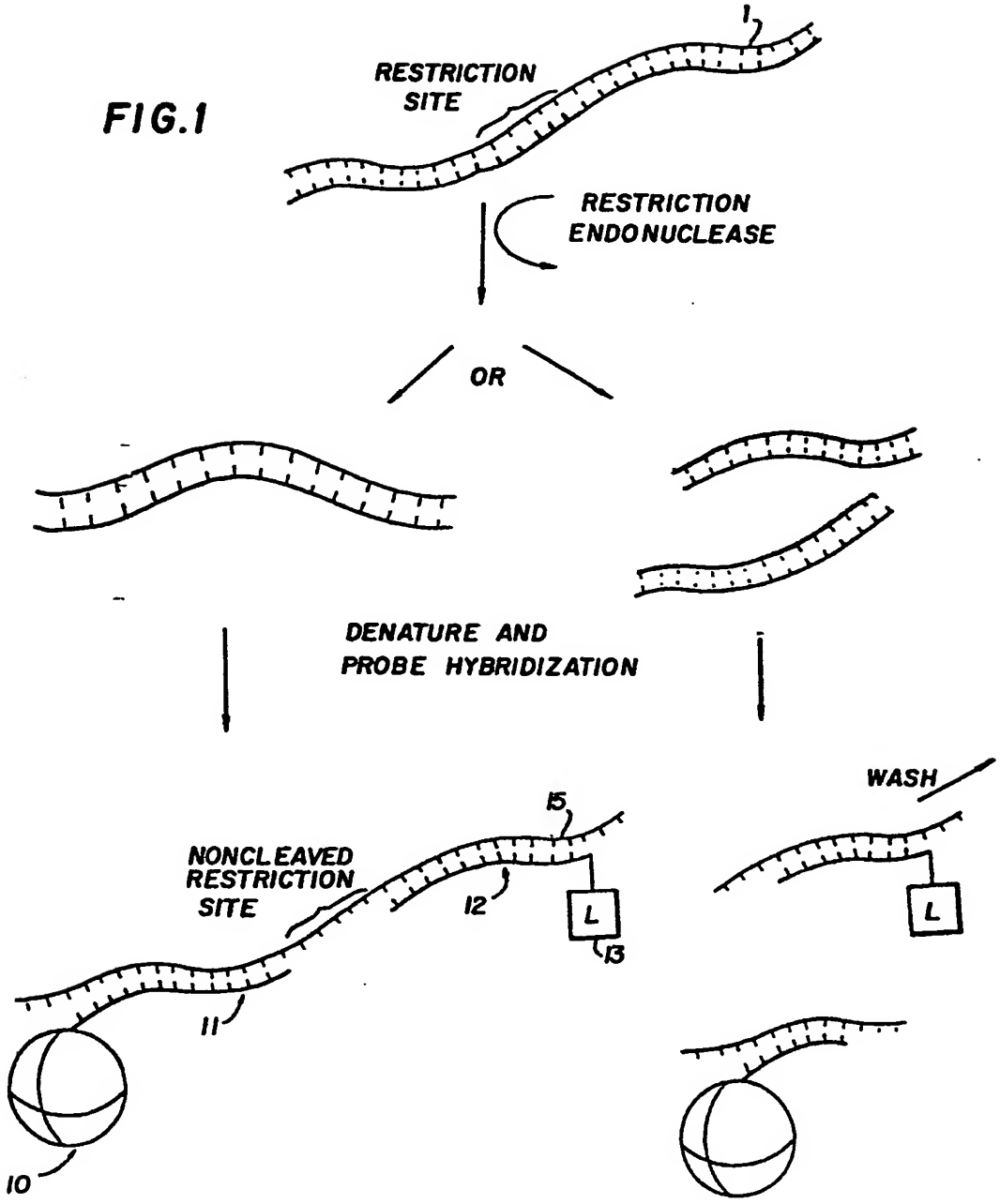
e) reacting said single stranded nucleic acid with said first and second insolubilized nucleic acid probes under conditions which permit hybridization to occur;

f) determining the presence of said specific base pair sequence on the basis of agglutination or changes in agglutination settling rates.

12. The method as provided in Claim 11 wherein said first and second nucleic acid probes are immobilized by attachment to a solid phase selected from the group consisting of microbeads, macrobeads, latex particles and metallic particles.

13. The method as provided in claim 11 or claim 12 wherein a decrease in agglutination settling rate is related to restriction endonuclease activity and the presence of the specific base pair sequence.

FIG.1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RESTRICTION SITE
RESTRICTION ENDONUCLEASE
OR
DENATURE AND PROBE HYBRIDIZATION
WASH
15
NONCLEAVED RESTRICTION SITE
12
L
13
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10
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